

FEBS Letters 353 (1994) 67–70

FEBS 14628

GDP/GTP exchange reaction-stimulating activity of Rabphilin-3A for *Rab3A* small GTP-binding protein

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Received 22 August 1994

Abstract Rabphilin-3A is a putative target protein for *Rab3A*, a small GTP-binding protein particularly implicated in neurotransmitter release. Rabphilin-3A interacts more preferentially with GTP-*Rab3A* than with GDP-*Rab3A*. Moreover, Rabphilin-3A shows a weak activity to stimulate the GTPase activity of *Rab3A* and a strong activity to inhibit the *Rab3A* GTPase-activating protein (GAP)-stimulated GTPase activity of *Rab3A*. Here, we show that Rabphilin-3A has another activity to stimulate the GDP/GTP exchange reaction of *Rab3A*. Rabphilin-3A may keep *Rab3A* continuously in the GTP-bound form by converting again GDP-*Rab3A*, which may be converted from GTP-*Rab3A* by *Rab3A* GAP, to GTP-*Rab3A*, until the function of *Rab3A* is accomplished.

Key words: Small G protein; *Rab3A*; Rabphilin-3A; GEP activity

1. Introduction

Rabphilin-3A is a putative target protein for *Rab3A* which is implicated in regulated secretion, particularly in neurotransmitter release ([1–5]; for reviews, see [6,7]). Rabphilin-3A is a single polypeptide with a M_r of 77,976 and 704 amino acids which interacts preferentially with GTP-*Rab3A* than with GDP-*Rab3A* [2]. Rabphilin-3A has moreover two repeated C2-like domains at its C-terminal portion which interacts with Ca^{2+} and phospholipid, particularly phosphatidylserine [8]. Northern and Western blot analyses have revealed that Rabphilin-3A is specifically expressed in neuron where it is highly concentrated on the synaptic vesicle, although Rabphilin-3A lacks a transmembrane segment [2,9]. The function of Rabphilin-3A has not been defined, but our current working model for the mode of action of *Rab3A* and Rabphilin-3A in neurotransmitter release is as follows: In the resting synapse, GTP-*Rab3A* binds to Rabphilin-3A on the synaptic vesicle which is translocated to the active zone where Ca^{2+} channel and its accessory proteins are clustered. When the presynapse is depolarized and Ca^{2+} influxes into the cytosol, the synaptic vesicle fuses with the presynaptic plasma membrane resulting in the neurotransmitter release into the synaptic cleft. This fusion may be initiated by the general docking/fusion machinery of the NSF²-SNAP-SNARE system ([10]; for reviews, see [11,12]). The *Rab3A*-Rabphilin-3A system may be involved in translocating and targeting the synaptic vesicle to the active zone. Moreover, it may serve as an inhibitor for the docking/fusion machinery of

the NSF-SNAP-SNARE system as described for synaptotagmin ([13]; for a review, see [14]). It could be speculated that Ca^{2+} influxed into the cytosol binds to Rabphilin-3A and synaptotagmin and releases the inhibitory function of these two proteins, finally allowing the NSF-SNAP-SNARE system to be employed. After the fusion, GTP-*Rab3A* is converted to GDP-*Rab3A* by the action of *Rab3A* GAP. Once GDP-*Rab3A* is produced, its affinity for Rabphilin-3A is reduced and moreover it becomes complexed with *Rab* GDI. This complex dissociates from the membrane to the cytosol. GDP-*Rab3A* translocated to the cytosol is converted to GTP-*Rab3A* by the action of *Rab3A* GRF or MSS4 and it again interacts with Rabphilin-3A on the synaptic vesicle. Thus, the *Rab3A*-Rabphilin-3A system plays an important role in neurotransmitter release.

In this model, there may be a mechanism for continuously keeping *Rab3A* in the GTP-bound form until its function is accomplished. We have previously found that Rabphilin-3A potentially has a weak activity to stimulate the intrinsic GTPase activity of *Rab3A* (GAP activity) and a strong activity to inhibit the *Rab3A* GAP-stimulated GTPase activity of *Rab3A* (GIP activity) [15]. These properties of Rabphilin-3A are consistent with its another biochemical property that it interacts more preferentially with GTP-*Rab3A* than with GDP-*Rab3A* [2,8]. On the basis of these observations, we have proposed that this GIP activity may keep *Rab3A* in the GTP-bound form until its function is accomplished, although the physiological function of the GAP activity of Rabphilin-3A is not known.

During the course of these studies on Rabphilin-3A, we have incidentally found that Rabphilin-3A potentially has another activity to stimulate the GDP/GTP exchange reaction of *Rab3A* (GEP activity). We will show here this GEP activity of Rabphilin-3A and also discuss the possible physiological function of this GEP activity of Rabphilin-3A.

2. Materials and methods

2.1. Materials and chemicals

The post-translationally lipid-modified and lipid-unmodified forms

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Abbreviations: NSF, N-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; GAP, GTPase-activating protein; GDI, GDP dissociation inhibitor; GRF, guanine nucleotide releasing factor; GIP, GAP inhibiting protein; GEP, GDP/GTP exchange protein; GTP γ S, guanosine 5'-(3-O-thio)triphosphate; G protein, GTP-binding protein; GDS, GDP dissociation stimulator.

of *Rab3A* were purified from the membrane and soluble fractions, respectively, of *Spodoptera frugiperda* cells (Sf9 cells) that were infected with the baculovirus carrying the *Rab3A* cDNA [16]. *Rab* GDI was purified from bovine brain cytosol as described previously [17]. [35 S]GTP γ S (44.4 TBq/mmol) was purchased from DuPont-New England Nuclear. [3 H]GDP (518 GBq/mmol) was purchased from Amersham Corp. Nitrocellulose filters (BA-85, 0.45 μ m pore size) were obtained from Schleicher & Schuell. Phosphatidylserine was obtained from Serdary Research Laboratories. A Superdex 200 HR 10/30 column and a Heparin Sepharose CL-6B column were from Pharmacia LKB Biotech Inc. Other materials and chemicals were obtained from commercial sources.

2.2. Expression of Rabphilin-3A in Sf9 cells and its purification

The full-length cDNA encoding Rabphilin-3A was inserted and expressed in Sf9 cells using the insect/baculovirus system. Rabphilin-3A was recovered in the 100,000 \times g precipitate and extracted from it by 2.5% sodium cholate. The extracted protein was purified by Heparin Sepharose CL-6B and Superdex 200 HR 10/30 column chromatographies as described [18].

2.3. Assay for GEP activity

The GEP activity of Rabphilin-3A was assayed by measuring either the dissociation of [3 H]GDP from [3 H]GDP-*Rab3A* or the binding of [35 S]GTP γ S to GDP-*Rab3A* by the filtration method using nitrocellulose filters as described previously [19].

2.4. Determination

Protein concentrations were determined with bovine serum albumin as a reference protein as described previously [20].

3. Results

Recombinant Rabphilin-3A stimulated the dissociation of [3 H]GDP from *Rab3A* and the binding of [35 S]GTP γ S to *Rab3A* in time- and dose-dependent manners (Figs. 1 and 2). The time courses and doses of Rabphilin-3A for these two reactions were similar. The dose of Rabphilin-3A giving half maximum dissociation of [3 H]GDP from *Rab3A* was about 0.8 μ M and the dose of Rabphilin-3A giving half maximum binding of [35 S]GTP γ S to *Rab3A* was also about 0.8 μ M. The doses of Rabphilin-3A necessary for the GEP activity were 5- to 10-fold higher than those necessary for the GAP and GIP activities

described previously [15]. In contrast, Rabphilin-3A did not stimulate the dissociation of [35 S]GTP γ S from *Rab3A* (data not shown). In these experiments, the lipid-modified form of *Rab3A* was used as a substrate for Rabphilin-3A. When the lipid-unmodified form of *Rab3A* was used, Rabphilin-3A also stimulated the dissociation of [3 H]GDP from *Rab3A*, but the velocity toward the lipid-unmodified form was about one-third of that toward the lipid-modified form (Fig. 3).

We have shown that Rabphilin-3A interacts with Ca^{2+} and phospholipid, particularly acidic phospholipid such as phosphatidylserine, at its C terminal portion, which includes the C2-like domains [8], and that neither Ca^{2+} nor phosphatidylserine affects the GAP or GIP activity of Rabphilin-3A [15]. Similarly, neither Ca^{2+} nor phosphatidylserine affected the GEP activity of Rabphilin-3A (data not shown).

We have previously shown that *Rab* GDI inhibits the dissociation of [3 H]GDP from the lipid-modified form of *Rab3A* but not that from the lipid-unmodified form [21]. Similarly, *Rab* GDI inhibited the Rabphilin-3A-induced dissociation of [3 H]GDP from the lipid-modified form of *Rab3A*, but not that from the lipid-unmodified form (Fig. 4).

4. Discussion

We have shown here that recombinant Rabphilin-3A has not only the GAP and GIP activities for *Rab3A* but also the GEP activity for it. Rabphilin-3A interacts with GDP-*Rab3A* for its GEP activity whereas it interacts with GTP-*Rab3A* for its GAP and GIP activities. We have previously shown that Rabphilin-3A interacts preferentially with GTP-*Rab3A* but also weakly with GDP-*Rab3A* [2]. This property of Rabphilin-3A is consistent with the present result that the doses of Rabphilin-3A necessary for its GEP activity are 5- to 10-fold higher than those necessary for its GAP and GIP activities.

We have also shown here that Rabphilin-3A is active as a GEP for both the lipid-modified and lipid-unmodified forms of *Rab3A*, but prefers the former form to the latter form. We have previously shown that one group of GEPs, such as *Sos* for the

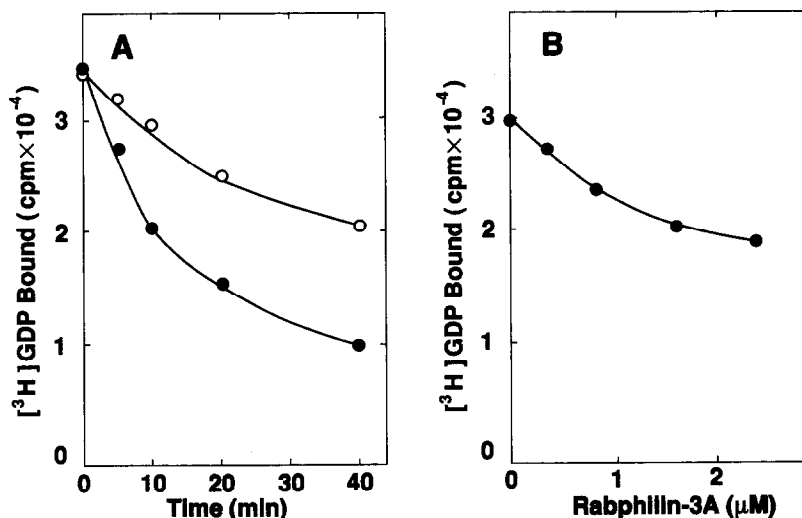


Fig. 1. Effect of Rabphilin-3A on the dissociation of [3 H]GDP from *Rab3A*. (A) Time course. [3 H]GDP-*Rab3A* was incubated for the indicated periods of time in the presence or absence of Rabphilin-3A (1.6 μ M). (●), in the presence of Rabphilin-3A; (○), in the absence of Rabphilin-3A. (B) Dose-dependent effect of Rabphilin-3A. [3 H]GDP-*Rab3A* was incubated for 10 min with the indicated amounts of Rabphilin-3A. The GEP activity of Rabphilin-3A was expressed as a decrease in the radioactivity trapped on the nitrocellulose filters in the presence of Rabphilin-3A compared with that in the absence of Rabphilin-3A. The results shown are representative of three independent experiments.

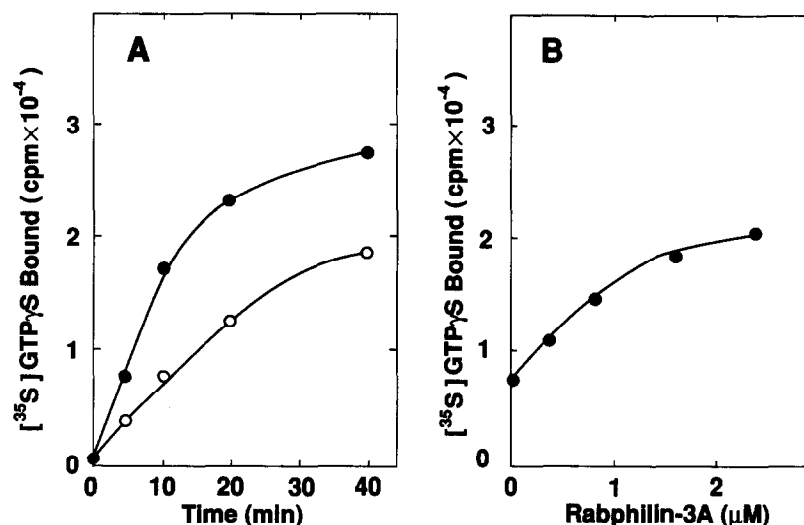


Fig. 2. Effect of Rabphilin-3A on the binding of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ to Rab3A. (A) Time course. Rab3A was incubated with $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ for the indicated periods of time in the presence or absence of Rabphilin-3A (1.6 μM). (●), in the presence of Rabphilin-3A; (○), in the absence of Rabphilin-3A. (B) Dose-dependent effect of Rabphilin-3A. Rab3A was incubated with $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ for 10 min with the indicated amounts of Rabphilin-3A. The GEP activity of Rabphilin-3A was expressed as an increase in the radioactivity trapped on the nitrocellulose filters in the presence of Rabphilin-3A compared with that in the absence of Rabphilin-3A. The results shown are representative of three independent experiments.

Ras family members [22], Cdc25 for the Ras family members [23], Dbl for the Rho family members, the Rac family members, and Cdc42 [24], and Rab3A GRF for Rab3A [25], are active on both the lipid-modified and lipid-unmodified forms of their respective substrate small G proteins but prefer much more the former form to the latter form, whereas another group of GEPs, such as Smg GDS for Ki-Ras, Rap1, the Rho family members, the Rac family members, and Cdc42 [24,26,27], Rho GDI for the Rho family members, the Rac family members, and Cdc42 [27,28], and Rab GDI for the Rab family members [21], absolutely require the lipid-modifications of their respective substrate small G proteins and are completely inactive on the lipid-unmodified form. In addition to these two groups of GEPs, we have recently shown that MSS4 is equally active on both the lipid-modified and lipid-unmodified forms of Rab3A [19].

We have previously shown that Rab GDI inhibits the Rab3A GRF-induced GDP/GTP exchange reaction of the lipid-modified form of Rab3A but not that of the lipid-unmodified form [25]. We have recently shown that Rab GDI inhibits the MSS4-induced GDP/GTP exchange reaction of the lipid-modified form of Rab3A but not that of the lipid-unmodified form [19]. Consistent with these observations, we have shown here that Rab GDI inhibits the GEP activity of Rabphilin-3A for the lipid-modified form of Rab3A, but not that for the lipid-unmodified form.

Rabphilin-3A has two repeated C2-like domains and interacts with Ca^{2+} and phospholipid, particularly acidic phospholipid such as phosphatidylserine, at these domains [8]. We have previously reported that neither Ca^{2+} nor phosphatidylserine affects the GAP or GIP activity of Rabphilin-3A [15]. We have shown here that neither Ca^{2+} nor phosphatidylserine affects the GEP activity of Rabphilin-3A. The physiological function of C2-like domains of Rabphilin-3A still remains unknown.

Finally, what is the physiological function of the GEP activity of Rabphilin-3A? One possibility is that this GEP activity

of Rabphilin-3A is an artifact just due to the physicochemical property. Another possibility is that the GEP activity of Rabphilin-3A functions under particular conditions. It could be speculated that Rab3A should be continuously kept in the GTP-bound form until its function is accomplished. For this purpose, GTP-Rab3A complexed with Rabphilin-3A may be protected from Rab3A GAP by the GIP activity of Rabphilin-3A. However, it would be possible that GTP-Rab3A may be converted to GDP-Rab3A by the action of Rab3A GAP or the intrinsic GAP activity of Rabphilin-3A before its function is accomplished. When this event occurs, Rabphilin-3A may

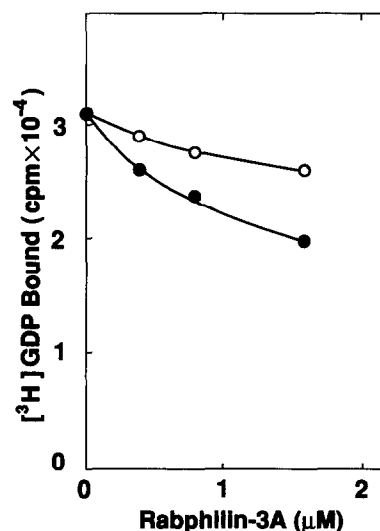


Fig. 3. The GEP activity of Rabphilin-3A for the lipid-modified and lipid-unmodified forms of Rab3A. The lipid-modified or lipid-unmodified form of $[^3\text{H}]\text{GDP}$ -Rab3A was incubated for 10 min in the presence of the indicated amounts of Rabphilin-3A. (●), the lipid-modified form; (○), the lipid-unmodified form. The results shown are representative of three independent experiments.

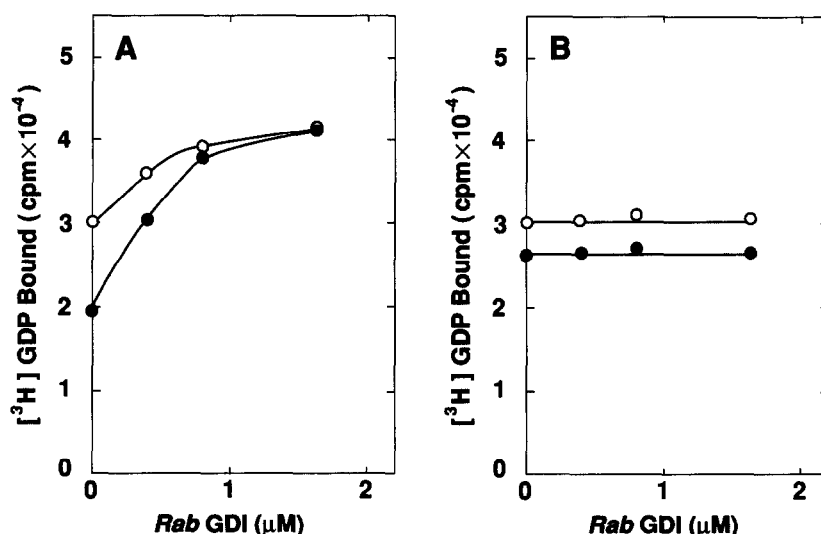


Fig. 4. Effect of Rab GDI on the GEP activity of Rabphilin-3A. The lipid-modified or lipid-unmodified form of [^3H]GDP-Rab3A was incubated for 10 min with the indicated amounts of Rab GDI in the presence or absence of Rabphilin-3A (1.6 μM). (A) The lipid-modified form; (B), the lipid-unmodified form. (●), in the presence of Rabphilin-3A; (○), in the absence of Rabphilin-3A. The results shown are representative of three independent experiments.

again return GDP-Rab3A to GTP-Rab3A by its intrinsic GEP activity before GDP-Rab3A becomes complexed with Rab GDI. Thus, Rabphilin-3A may possess the intrinsic GEP activity as a self-guarantee for keeping Rab3A in the GTP-bound form. Further studies are essential for understanding the physiological significance of the GEP, GAP, and GIP activities of Rabphilin-3A.

Acknowledgements: This investigation was supported by grants-in-aid for Scientific Research and for Cancer Research from the Ministry of Education, Science, and Culture, Japan (1994), by grants-in-aid for Abnormalities in Hormone Receptor Mechanisms and for Aging and Health from the Ministry of Health and Welfare, Japan (1994), by grants from the Yamanouchi Foundation for Research on Metabolic Disease (1994), Uehara Memorial Foundation (1994), and Nissan Science Foundation (1994).

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